

REMARKS

Claims 72-95 are pending in the present application and at issue.

It is respectfully submitted that the present amendment presents no new issues or new matter and places this case in condition for allowance. Reconsideration of the application in view of the above amendments and the following remarks is requested.

I. The Rejection of Claims 72-90 under 35 U.S.C. 103

The Office maintained the rejection of claims 72-90 under 35 U.S.C. 103 as being unpatentable over Lischnig et al. (Biotechnology Letters, Vol. 15, No. 4, pp. 411-414 (1993)) or Gomes et al. (Appl. Microbiol. Biotechnol., Vol. 39, pp. 700-707 (1993)) or Alam et al. (Enzyme Microb. Technol., Vol. 16, pp. 298-302 (1994)) and Haarasilta et al. (U.S. Patent No. 5,314,692) and Hazlewood et al. (WO 93/25693). This rejection is respectfully traversed.

As mentioned in the prior response, none of the cited references teach or suggest the use of thermostable xylanases in animal feed compositions, as claimed herein.

Moreover, none of the cited references teach or suggest that there would be any advantage to using a thermostable xylanase over a thermolabile xylanase in animal feed. As explained in the prior responses, animal feeds comprising a thermostable xylanase of Family 11 according to the present invention have significantly improved feed utilization over animal feeds comprising other xylanases.

In response to Applicants' showing of surprising and unexpected results, the Office stated:

Applicants next argue against the rejection using the doctrine of 'surprising and unexpected results'. Applicants refer to a comparative experiment [in Example 8] in which they compare a commercial enzyme with that of the invention and argue that the animal feed comprising the commercial enzyme at a dose of 400 FXU/kg gave a % fat digestion in the range of 72.1-74.3 [sic, "in the range of 72.1-74.3" should be replaced with "72.4"], while the animal feed comprising the present invention gave a % fat digestion in the range of 72.1-74.3 even though the xylanase was dosed at 100 or 200 FXU/kg and therefore the instant invention demonstrated superior property not predicted by prior art and that these results are surprising and unexpected. Examiner finds it difficult to accept the above results as surprising or unexpected. This is because it would be well within the level of skill of those practicing the art that decrease in viscosity will lead to better absorption and better digestion of feed ingredients. As pointed by the applicants themselves, it was well known in the art through the teachings of Anison et al. that high viscosity reduces the apparent metabolizable energy of feed ingredients. Therefore, this aspect of the invention cannot be considered as surprising or unexpected of the xylanase enzyme in the feed.

This is respectfully traversed.

The Office is correct that the results in Example 8 show that the use of Bio-Feed 'Plus, a commercially-available xylanase preparation derived from *Humicola insolens*, at a dose of 400 FXU/kg, gave a % fat digestion of 72.4, whereas the animal feeds comprising a xylanase of the present invention gave a % fat digestion in the range of 72.1-74.3 even though the xylanase of the present invention was dosed at 100 or 200 FXU/kg (i.e., one quarter or one-half, respectively, of the Bio-Feed Plus). Thus, the results show that the xylanase of the present invention has a 2-4 fold improvement over the commercially-available xylanase. In his second declaration of March 18, 2005 [hereinafter, "Supplemental Declaration"], Mr. Pettersson states in paragraph 8 that "these results are surprising and unexpected."

The Office merely argues that it is difficult to accept that these results are surprising and unexpected due to the fact that it is well known that a decrease in viscosity will lead to better absorption of feed ingredients etc. However, the decrease in viscosity is merely a likely reason why xylanases may improve the fat digestion. It is not a reason why the xylanases of the invention are surprisingly better than the prior art: The Office has not provided any evidence that a 2-4 fold lower dosage of the xylanases of the invention would provide a corresponding improvement in % fat digestion over the prior art xylanase. Thus, these results are surprising and unexpected.

The Office also responded to the results set forth in paragraphs 3-5 of Mr. Pettersson's Supplemental Declaration under 37 C.F.R. 1.132. Specifically, the Office states:

In summary Mr. Pettersson is arguing that the value 2.02 obtained for the feed comprising xylanase from *A. aculeatus* is less significant than the value 1.97 obtained for the feed comprising the xylanase from *T. lanuginosus*. Such a declaration is very hard for the Examiner to accept since it defies scientific explanation. It is highly perplexing to the Examiner as to how the two values 2.02 and 1.97 (differing by only 0.05) can be considered statistically significant without any supporting data such as sample size, degrees of freedom or even the bare minimum of a 'p' value for the data obtained. Actually, the values appear to be within the experimental error of an experiment involving animals.

This is respectfully traversed.

It appears that the Office has misunderstood Mr. Pettersson's Supplemental Declaration. Mr. Pettersson expressly states that the results obtained with the xylanase of the present invention (1.97) is statistically significant (i.e., outside the experimental error) when compared with the control, whereas the results obtained with the xylanase of Family 10 (2.02) is

not statistically significant (*i.e.*, within the experimental error) when compared with the control. Mr. Pettersson does not make any statements on the difference between the two values 1.97 and 2.02. In paragraph 6, Mr. Pettersson concludes:

[T]hese results show that animal feeds comprising the *Thermomyces lanuginosus* xylanase result in a statistically significantly improved feed utilization. A similar conclusion cannot be drawn as regards animal feeds comprising the *Aspergillus aculeatus* xylanase. Furthermore, the *Thermomyces lanuginosus* xylanase has a statistically significant influence on the viscosity of the digesta, which is highly surprising, given the fact that family 11 xylanases are not recognized to be strong viscosity reducers. These results are surprising and unexpected.

There is no evidence contradicting Mr. Pettersson's conclusion.

Applicants also enclose a copy of Choct et al., British Journal of Nutrition, 2004, 92: 53-61, of which Mr. Pettersson is a co-author. This article describes the entire study underlying Mr. Pettersson's Supplemental Declaration, including supporting data such as sample size. A total of 288 male broiler chickens were used, *i.e.*, 8 treatments, 6 cages per treatment, and 6 chickens per cage (see pages 54 and 55, line 1 of the first full paragraph of column 1). Please note that the results obtained with the Family 10 xylanase set forth in the article is 2.04 (see Table II on page 56, "xylanase C"), not the value of 2.02 described in Mr. Pettersson's Supplemental Declaration which came from preliminary raw data.

For the foregoing reasons and the reasons set forth in the prior responses, Applicants submit that the claims overcome this rejection under 35 U.S.C. 103. Applicants respectfully request reconsideration and withdrawal of the rejection.

II. The Rejection of Claims 72-90 under the Doctrine of Obviousness-Type Double Patenting

The Office maintained the rejection of claims 72-90 under the doctrine of obviousness-type double patenting as being unpatentable over claims 1-17 of U.S. Patent No. 6,245,546.

As mentioned in the prior response, Applicants will submit a terminal disclaimer upon an indication of allowable subject matter.

III. Conclusion

In view of the above, it is respectfully submitted that all claims are in condition for allowance. Early action to that end is respectfully requested. The Examiner is hereby invited to

contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

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Elias J. Lambiris, Reg. No. 33,728
Novozymes North America, Inc.
500 Fifth Avenue, Suite 1600
New York, NY 10110
(212) 840-0097

A comparison of three xylanases on the nutritive value of two wheats for broiler chickens

M. Choct¹*, A. Kocher¹, D. L. E. Waters^{1,2}, D. Pettersson³ and G. Ross^{4,5}

¹School of Rural Science and Agriculture, University of New England, Armidale, NSW 2351, Australia

²Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW 2480, Australia

³Novozymes A/S, 2880 Bagsværd, Denmark

⁴Novozymes Australia Pty Ltd, PO Box 4942, North Rocks, NSW 2151, Australia

⁵DSM Nutritional Products, Frenchs Forest, NSW 2086, Australia

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Three xylanase products, xylanase A derived from *Thermomyces lanuginosus*, xylanase B from *Humicola insolens* and xylanase C from *Aspergillus aculeatus*, were examined for their effects on the nutritive value of wheat. The study investigated the effects of enzyme addition to broiler diets based on a low-metabolisable-energy (ME) wheat and a normal-ME wheat, with the emphasis on changes in composition of the NSP along the digestive tract in broiler chickens. There were significant ($P < 0.01$) enzyme and wheat effects on the apparent ME, but there was no wheat \times enzyme interaction on apparent ME. Weight gain and feed conversion efficiency (except xylanase C for normal wheat) were also significantly ($P < 0.01$) increased by the xylanases. The enzymes differed in their effect on a number of important nutritional parameters. Xylanase A reduced ($P < 0.05$) the excreta moisture level from 77.1 % in birds fed the normal-ME wheat control to 73.4 % and from 77.4 % in those fed the low-ME wheat to 73.0 %. The other two enzymes did not affect excreta moisture levels. The digesta viscosity in the duodenum, jejunum and ileum of birds fed both types of wheat was reduced ($P < 0.01$) by xylanases A and C, but was increased ($P < 0.01$) in the jejunum and ileum by xylanase B. The digestibility of the soluble NSP was mostly negative in the small intestine. The digestibility of the insoluble NSP differed ($P < 0.01$) between the two wheats, with those in the low-ME wheat being more digestible (14 v. 28 %). When supplemented with xylanase A, the ileal digestibility of the insoluble NSP in the normal- and low-ME wheats was increased by 28 and 42 %, respectively. Xylanases B and C did not affect the digestibility of the insoluble NSP regardless of wheat type. It may be concluded that wheats with low or normal ME values vary in their responses to xylanase supplementation. Apart from having an elevated level of soluble NSP, low-ME wheat may also contain insoluble NSP, which in the present study appeared to be more easily degradable in the gut of the chicken.

Poultry: Enzymes: Non-starch polysaccharides: Nutritive value of wheat

Nutritionists are often not able to test raw ingredients and establish a true feeding value in advance of use in commercial feed formulations. Variation in raw ingredients accounts for 30 % of the variation in the finished product and creates multiplicative effects together with weighing errors (Fawcett & Webster, 1996). Subsequently, the diet may not reach the required specifications, which can result in sub-optimal performance and economic loss.

The apparent metabolisable energy (AME) values of Australian wheat have been reported to range from 10.35 to 15.9 MJ/kg DM (Mollah *et al.* 1983; Rogel *et al.* 1987). The so-called 'low-metabolisable energy (ME) wheats' are defined as wheats having AME values under 13 MJ/kg DM for poultry (Mollah *et al.* 1983). Approximately 30 % of Australian wheats fall into this category. When low-ME wheats are included as the main energy source in broiler diets, sticky and watery droppings will

appear immediately, followed by growth depression and poor feed efficiency. Wheat contains 5–8 % arabinoxylans (pentosans, consisting of the monosaccharides arabinose and xylose linked in β 1–4 linkages with arabinose side chains through the O2 and/or O3 positions of the backbone), up to 1 % of β -glucans and 2–3 % of cellulose. These NSP are believed to be the major cause of the low-ME wheat phenomenon (Annison & Choct, 1991). The viscous nature of these polysaccharides has a direct influence on nutrient absorption as well as interacting with the gut microflora and modifying the physiological function of the gut. High levels of soluble NSP raise digesta viscosity in the intestine of chickens leading to reduced starch, protein and lipid digestion (Choct & Annison, 1990; Annison, 1991; Philip *et al.* 1995). It is well documented that the addition of commercial feed enzymes to wheat-based broiler diets generally results in

Abbreviations: AME, apparent metabolisable energy; ME, metabolisable-energy; VFA, volatile fatty acid.

* Corresponding author: Professor Mingan Choct, fax +61 02 6773 3050, email mchoct@une.edu.au

a significant improvement in performance and a reduction in the occurrence of wet and sticky droppings (Annison, 1992; Choct *et al.* 1994). Xylanases reduce the intestinal viscosity of birds by degradation of soluble arabinoxylans. The degree of degradation of these polymers in the intestinal tract of broilers is unknown. When a depolymerised pentosan preparation was added to a broiler diet its anti-nutritive effect largely disappeared (Choct & Annison, 1992a). But the digesta viscosity of birds fed this diet was significantly higher in comparison with birds fed a sorghum-based control diet. This finding suggests that a reduction in viscosity might not be the only parameter of importance influencing the AME value of wheat.

Detailed knowledge of the substrates and their breakdown pattern in the intestine of chickens is required in order to fully comprehend the effects of wheat NSP. The present study was carried out to test the hypothesis that the nutritive value of wheat for poultry is related to the viscous nature of the soluble NSP as well as the encapsulation of nutrients by cell-wall components of the grain, namely the insoluble arabinoxylans. Thus, enzymes that have affinity for both these fractions could be superior to one that reduces viscosity alone or that attacks mainly the cell walls.

Materials and methods

Bird husbandry

A total of 288 male broiler chicks (1-d-old; Cobb strain) were obtained from the Baiada Hatchery, Kootingal, New South Wales and were raised on a commercial broiler starter diet (crumbles, Ridley AgriProducts, Tamworth, NSW, Australia), containing 12.5 MJ ME/kg and a minimum of 20% crude protein, in standard chick brooders for 24 d. During experimentation, all birds were transferred to wire-mesh metabolism cages situated in climate-controlled rooms. All experimental diets were cold-pelleted. The diets

were then offered to the birds *ad libitum* and water was freely available. Birds were grown under continuous fluorescent lighting. The animal ethics committee of the University of New England approved the present study. Health and husbandry practices complied with the *Code of Practice for the Care and Use of Animals for Scientific Purposes* (National Health & Medical Research Council, 1990) for the Commonwealth of Australia and the *Australian Model Code of Practice for the Welfare of Animals* (Standing Committee on Agriculture and Resource Management, 1995).

Diet

Two wheats with known low or normal ME values were chosen for the present study based on a preliminary study reported by Choct & Kocher (2000). The low-ME wheat had a measured AME value of 12.5 MJ/kg DM at the time of harvest, whereas the normal wheat had an AME value of 11.5 MJ/kg DM.

All diets were formulated using the ingredients and proportions shown in Table 1. First, the minor ingredients were mixed in a small rotary mixer and were then pooled with the major ingredients for thorough mixing. All experimental diets were identical in composition. Three xylanase products differing in their modes of action were selected for the present experiment. The enzymes were: xylanase A, which was purported to have affinity for both soluble and insoluble NSP; xylanase B, which was mainly known to solubilise insoluble NSP; xylanase C, which under laboratory conditions mainly reduced viscosity (Dr Dan Pettersson, Novozymes AS, Bagsvaerd, Denmark, personal communication.). Each tonne of feed contained 200 g xylanase A (activity, 1000 fungal xylanase unit/g; commercial name, Bio-Feed Wheat or Ronozyme WX; derived from *Thermomyces lanuginosus*), 250 g xylanase B (activity, 800 fungal xylanase unit/g; commercial name, Bio-Feed Plus or Ronozyme W; derived from *Humicola insolens*) or 426 g xylanase C (activity, 469 fungal xylanase unit/g; an

Table 1. Composition of the experimental diets (g/kg)

Ingredients	Diets							
	1	2	3	4	5	6	7	8
Normal-ME wheat (Cumawong)	784	784	784	784	—	784	784	784
Low-ME wheat (Harvey)	—	—	—	—	784	784	784	784
Casein	149	149	149	149	149	149	149	149
Dicalcium phosphate	19.6	19.6	19.6	19.6	19.6	19.6	19.6	19.6
Limestone	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8
DL-Methionine	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
Vitamin-mineral pre-mix*	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Salt	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Choline chloride	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Marker†	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Xylanase A‡	—	0.2	—	—	—	0.2	—	—
Xylanase B‡	—	—	0.26	—	—	—	0.26	—
Xylanase C‡	—	—	—	0.426	—	—	—	0.426

ME, metabolisable energy.

*The active ingredients contained in the vitamin-mineral premix were as follows (per kg): retinol 3.03 mg, cholecalciferol 0.08 mg, all-rac- α -tocopherol acetate 20 mg, menadione 6.3 mg, riboflavin 8 mg, pyridoxine hydrochloride 6 mg, biotin 0.01 mg, niacin 30 mg, thiamin 1.5 mg, D-calcium pantothenate 15 mg, folic acid 2 mg, ethoxyquin 125 mg, Mn 75 mg, Fe 20 mg, Cu 5 mg, I 1 mg, Co 0.3 mg, Se 0.5 mg, Mo 0.16 mg, cyanocobalamin 0.13 mg.

† Celite (acid-insoluble ash) was obtained from Celite Corporation (Lompoc, CA, USA).

‡ For details of xylanases see p. 64.

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experimental product derived from *Aspergillus aculeatus*). The activities were determined by Novozymes AS of Denmark. Celite was included to all diets at 20 g/kg as a marker for the calculation of nutrient digestibility in the small intestine.

Apparent metabolisable energy bioassay

On day 24, chickens were weighed in groups of six and transferred to forty-eight metabolism cages. The first 3 d served as an adaptation period and for the last 4 d excreta were collected daily, dried overnight in a forced-air oven at 80°C and pooled for the determination of gross energy. The amount of feed consumed during the adaptation period and the excreta collection period was recorded. On completion of the 7 d AME bioassay period the birds were weighed in groups following their killing by cervical dislocation. The body cavity was opened and the contents of the duodenum, jejunum (from duodenum to the Meckel's diverticulum), ileum (from the Meckel's diverticulum to 40 mm above the ileo-caecal junction) and caecum were collected. Digesta of birds from each cage was pooled for the same gut section and fresh weights recorded. The supernatant fractions and the pellets were immediately separated by centrifugation (12 000 g, 15 min) and frozen at -21°C for future analyses.

The AME value of the major dietary ingredient (cereal) was obtained by using a pre-determined AME value (20.1 MJ/kg DM) for casein (Annison *et al.* 1994). The calculation of the AME of the grain was as follows:

$$\text{AME}_{\text{grain}} = (\text{AME}_{\text{dcl}} - \text{AME}_{\text{casein}}) \times \text{dry casein level} / \text{dry grain level.}$$

Laboratory analyses

Digesta viscosity. Approximately 2 g fresh digesta were centrifuged (12 000 g, 10 min at 20°C) and the viscosity was determined on 0.5 ml supernatant fraction using a Brookfield Model DV-III Rheometer (Brookfield Engineering Laboratories Corp., Stoughton, MA, USA) at 25°C with a CP40 cone and shear rate of 5–500/s. The samples did not exhibit shear thinning at these shear rates.

Volatile fatty acids. For caecal volatile fatty acid (VFA) determination, 3 g fresh sample were diluted with 0.1 M-sulfuric acid (3 ml) and thoroughly mixed. The sample was centrifuged (12 000 g, 15 min). To a sample of 1 ml supernatant fraction, 0.1 ml of a reference VFA (caproic acid) were added. The VFA were distilled using Thundberg tubes. The concentration of VFA was quantified using a Hewlett Packard 427 gas-liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA). For ileal VFA determination, the fresh sample was centrifuged (12 000 g, 15 min). To a sample of 1.3 ml supernatant fraction, 0.1 ml of a reference VFA (caproic acid) and 0.5 M-sulfuric acid (0.1 ml) were added. The VFA were distilled using Thundberg tubes. The samples were treated with two drops of 10 M-sodium hydroxide and concentrated to dryness in a vacuum desiccator. The VFA salts were dissolved

in 0.5 M-sulfuric acid (0.1 ml) and were quantified using a Hewlett Packard 427 gas-liquid chromatograph. The gas chromatograph was fitted with a glass column packed with *o*-phosphoric acid (1.5%, w/w) and polypropylene glycol sebacate (15.5%) on chromosorb 'W', acid-washed, 60–80 mesh. Operating conditions using a flame ionisation detector were: column temperature, 135°C; detector temperature, 180°C; carrier gas (N₂) flow, 25 cm³/min; H₂ flow, 25 cm³/min; air flow, 220 cm³/min.

Proximate analyses. DM was determined using a drying oven at 105°C for 6 h. Gross energy contents of diets and excreta were determined using a DDS 500 isoperibol calorimeter (Digital Data Systems, Johannesburg, South Africa). Acid-insoluble ash in the jejunal and ileal digesta was determined following the method described by Choct & Annison (1992b). The N content of the diets and ileal digesta was determined using a LECO FP-2000 automatic analyser (LECO Corp., St Joseph, MI, USA). The protein contents were calculated using a multiplication factor of 5.7. Experimental diets and ileal digesta were analysed for soluble NSP, insoluble NSP and free sugars by the Uppsala method (Theander & Westerlund, 1993).

Statistical analysis

All data were analysed using either Base SAS® software (SAS Institute, Inc., 1988) or Statgraphics (Manugistics, Inc., 2000). ANOVA (by the general linear model procedure) was used to determine the significance of the main effects and any interactions, where appropriate. Duncan's multiple-range test was used to separate means when significant main effects ($P < 0.05$) were detected by ANOVA. When significant effects ($P < 0.05$) of interaction were evident from ANOVA, *t* tests (by least-squares means procedure) were used to separate least-squares means.

Results*Bird performance, apparent metabolisable energy and excreta moisture*

Xylanase supplementation increased ($P < 0.001$) the weight gain of birds compared with the unsupplemented controls regardless of wheat type. The feed conversion ratio followed a similar pattern, except for that in birds fed normal wheat with xylanase C. The AME value of the unsupplemented wheat was higher in the present study compared with that reported in the preliminary study (Choct & Kocher, 2000). However, there remained a difference of 1 MJ between the two wheats, with the low-ME wheat having an AME value lower than 13 MJ/kg. The addition of enzymes to the normal-ME wheat had no effect on the AME values. In contrast, the addition of xylanases A and C to low-ME wheat significantly ($P < 0.05$) improved the AME value. Xylanase B had no effect on the AME value of the low-ME wheat. Xylanase A significantly ($P < 0.05$) reduced the moisture content of the excreta of birds fed both normal- and low-ME wheats, whereas the effect of xylanase B and xylanase C on excreta moisture was not significant. All the data are presented in Table 2.

Table 2. Growth rate, feed conversion ratio (FCR), apparent metabolisable energy (AME) of wheat and excreta moisture content of broiler chickens fed on diets containing normal-metabolisable energy (ME) wheat and low-ME wheat with and without enzyme supplementation[†]
(Mean values and standard deviations)

Wheat	Enzyme	Weight gain (g/bird/per week)		FCR (g feed/g gain)		AME of wheat (MJ/kg DM)		Excreta moisture (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Normal-ME	Control	345 ^c	33	2.158 ^b	0.110	13.7 ^{ab}	0.5	77.1 ^a	1.6
Normal-ME	Xylanase A	392 ^{ab}	20	1.965 ^{bo}	0.055	14.5 ^{ab}	0.4	73.4 ^b	1.9
Normal-ME	Xylanase B	395 ^{ab}	29	2.005 ^{bo}	0.104	13.9 ^{ab}	0.9	76.1 ^{ab}	3.6
Normal-ME	Xylanase C	383 ^{ab}	33	2.035 ^{bo}	0.107	14.2 ^{ab}	0.7	75.0 ^{ab}	2.2
Low-ME	Control	369 ^a	24	2.089 ^{ab}	0.137	12.7 ^a	0.6	77.4 ^a	3.1
Low-ME	Xylanase A	410 ^a	31	1.910 ^a	0.055	13.6 ^{ab}	0.9	73.0 ^b	2.4
Low-ME	Xylanase B	385 ^{ab}	12	2.015 ^{bo}	0.104	13.3 ^{bo}	0.4	75.1 ^{ab}	1.6
Low-ME	Xylanase C	401 ^{ab}	23	1.923 ^c	0.121	13.9 ^{ab}	0.8	74.7 ^{ab}	2.4
Probability of greater F value in ANOVA									
Wheat		NS		NS		***		NS	
Enzyme		***		***		***		***	
Wheat x enzyme		NS		NS		NS		NS	

^{a,b,c}Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

*** $P<0.001$.

† For details of diets and procedures, see Table 1 and p. 54.

The enzymic degradation patterns of non-starch polysaccharides along the gut

The low-ME wheat (Harvey) and the normal-ME wheat (Currawong) were analysed for NSP contents. The low-ME wheat contained 17.6 g soluble NSP/kg DM and 75.6 g insoluble NSP/kg DM, whereas the normal-ME wheat contained 16.1 g soluble NSP/kg DM and 71.9 g insoluble NSP/kg DM. The main difference between the sugar composition of the two wheats was in the contents of arabinose and xylose, where the low-ME wheat contained proportionally more of these sugars.

The xylanases did not increase the level of free sugars (sugars with one to twelve monosaccharide units) both in the jejunum and the ileum regardless of wheat type. However, xylanase C decreased the level of free sugars in the jejunum of birds fed the low-ME wheat, compared with xylanases A and B.

NSP concentrations in the jejunum and ileal digesta were not affected by wheat type; however, the xylanases significantly altered the concentrations of NSP in comparison with the unsupplemented control diet. Thus, xylanase A significantly ($P<0.05$) increased the jejunum soluble NSP concentrations in birds fed both types of wheat, and ileal soluble NSP concentration in birds fed the low-ME wheat. Xylanase B increased ($P<0.05$) the jejunum and ileal soluble NSP concentrations in birds fed the low-ME wheat and the ileal soluble NSP concentration in birds fed the normal wheat, compared with those in birds fed the unsupplemented control. This led to a significant wheat x enzyme interaction. Xylanase A also decreased ($P<0.01$) the insoluble NSP concentration in the ileal digesta of birds fed both types of wheat, and xylanase C reduced ($P<0.01$) the total NSP level in the ileal digesta of birds fed the low-ME wheat. The data are shown in Table 3.

Neutral sugar composition of non-starch polysaccharides in the jejunum and ileum

The ability to release free sugars by the three xylanases differed significantly ($P<0.05$), with xylanases A and C releasing higher amounts of arabinose and xylose (the two main sugars of wheat NSP, the arabinoxylans) both in the jejunum and the ileal digesta of birds fed the normal-ME wheat. Xylanase B did not release free sugars in the jejunum and the ileum. Xylanases A and B elevated ($P<0.05$) the level of soluble NSP in the jejunum and ileum, whereas xylanase B had no effect on the jejunum and ileal soluble arabinoxylan levels for both wheats (Table 4).

For birds fed the normal-ME wheat, the insoluble arabinoxylan level in the jejunum was lowered ($P<0.05$) by xylanases A and B, whereas that in the ileum was decreased ($P<0.05$) only by xylanase A. Xylanase C did not have any effect on the insoluble arabinoxylan level regardless of the gut sections (Table 4). For birds fed low-ME wheat, however, the addition of xylanases A and B to the diet resulted in a numerical reduction in insoluble arabinoxylans in the jejunum and a significant ($P<0.05$) reduction in the ileum. Xylanase C reduced the insoluble arabinose concentration in the ileum compared with the control (Table 5).

Digesta viscosity along the gut

Both wheat type and xylanase supplementation had significant ($P<0.001$) effects on digesta viscosity in all sections of the intestine (Table 6). There was a highly significant ($P<0.001$) wheat x xylanase interaction on jejunum and ileal viscosity. Thus xylanases A and C reduced ($P<0.05$) digesta viscosity in the duodenum and jejunum for both wheats, but viscosity reduction in the ileal digesta was

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Table 3. Amounts of free sugars, soluble and insoluble NSP (sNSP and iNSP), and total NSP in jejunal and ileal digesta of broiler chickens fed on diets containing normal-metabolisable energy (ME) wheat and low-ME wheat with and without enzyme supplementation (mg/g marker)[†] (Mean values)

Wheat	Enzyme	Jejunum				Ileum			
		Free sugars	sNSP	iNSP	Total NSP	Free sugars	sNSP	iNSP	Total NSP
Normal-ME	Control	2010.2 ^{ab}	487.5 ^a	2527.2 ^a	5025.0 ^a	878.9 ^{nb}	509.5 ^{cd}	2349.5 ^{ab}	3737.9 ^{bcd}
Normal-ME	Xylanase A	2039.0 ^a	640.5 ^{ab}	2206.5 ^{ab}	4888.0 ^a	1019.3 ^a	547.4 ^c	1966.5 ^b	3533.2 ^{cd}
Normal-ME	Xylanase B	1971.9 ^{ab}	817.4 ^{cb}	2001.7 ^b	4488.2 ^a	1010.6 ^a	807.2 ^a	2500.1 ^a	4317.8 ^a
Normal-ME	Xylanase C	1929.8 ^{ab}	513.8 ^{ba}	2548.0 ^a	4906.9 ^a	886.6 ^{nb}	539.9 ^a	2482.5 ^a	3909.0 ^{abcd}
Low-ME	Control	1579.4 ^b	492.1 ^a	2637.6 ^a	4709.1 ^a	941.2 ^{nb}	509.2 ^{cd}	2609.5 ^a	4060.0 ^{ab}
Low-ME	Xylanase A	1915.1 ^{ab}	705.3 ⁿ	2416.3 ^{ab}	5036.8 ^a	1002.3 ^a	610.0 ^b	1991.2 ^b	3603.5 ^{bcd}
Low-ME	Xylanase B	2014.4 ^{nb}	715.5 ^a	2497.6 ^a	5227.5 ^a	980.9 ^a	809.0 ^a	2235.3 ^{ab}	4005.2 ^b
Low-ME	Xylanase C	1840.7 ^{nb}	471.9 ^a	2388.4 ^{nb}	4701.0 ^a	749.3 ^b	464.6 ^d	2221.5 ^{ab}	3435.4 ^a
Probability of greater <i>F</i> value in ANOVA									
Wheat		NS	NS	NS	NS	NS	NS	NS	NS
Enzyme		NS	***	NS	NS	NS	***	**	NS
Wheat x enzyme		NS	NS	NS	NS	NS	*	NS	NS

^{a,b,c,d} Values within a column with unlike superscript letters were significantly different ($P<0.05$).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

† For details of diets and procedures, see Table 1 and p. 54.

only significant for diets containing the low-ME wheat. In contrast, the addition of xylanase B to the unsupplemented control diets increased intestinal viscosity throughout the intestine. In diets with low-ME wheat the increases were significant ($P<0.05$) in all sections of the intestine.

Digestibility of non-starch polysaccharide fractions

The digestibility of soluble NSP was mostly negative, except in birds fed low-ME wheat supplemented with xylanase B. The negative effect of enzymes on the digestibility of soluble NSP both in the jejunum and ileum differed, with xylanase B producing a highly negative ($P<0.05$) ileal digestibility value compared with the other diets regardless of wheat type (Table 7). Both the jejunal and ileal digestibility values for the insoluble

NSP were significantly ($P<0.01$) higher for the low-ME wheat than the normal wheat. Xylanase A resulted in numerically higher digestibility values for the ileal insoluble NSP. There was a significant ($P<0.01$) wheat x enzyme interaction on the ileal digestibility of soluble NSP, with xylanase B giving a negative digestibility for the normal wheat and a positive digestibility value for the low-ME wheat.

Volatile fatty acid production in the ileum and caecum

The addition of enzymes to diets based on the normal-ME wheat had no effect on the production of VFA in the ileum or caecum (Table 8). In diets with low-ME wheat the addition of xylanases A and C reduced ($P<0.05$) the VFA concentration in the ileum.

Table 4. Main neutral sugar composition in the jejunal and ileal digesta of broiler chickens fed diets containing normal-metabolisable energy (ME) wheat with and without enzyme supplementation (g/kg marker)^{*} (Mean values)

	Enzyme	Jejunum			Ileum		
		Ara	Xyl	Glc	Ara	Xyl	Glc
Free sugars							
Normal-ME wheat	Control	9.2 ^c	5.8 ^a	1762.8 ^a	6.2 ^a	3.9 ^c	695.0 ^a
Normal-ME wheat	Xylanase A	45.1 ^a	115.6 ^a	1635.3 ^a	53.9 ⁿ	138.2 ^a	652.5 ⁿ
Normal-ME wheat	Xylanase B	10.8 ^c	11.8 ^a	1701.5 ^a	10.6 ^{ba}	11.8 ^{ba}	788.4 ^a
Normal-ME wheat	Xylanase C	28.6 ^b	67.9 ^b	1611.7 ^a	21.9 ^b	45.0 ^b	632.7 ^a
Soluble NSP							
Normal-ME wheat	Control	142.3 ^b	215.0 ^b	83.2 ^b	141.2 ^a	224.4 ^b	94.6 ^{ab}
Normal-ME wheat	Xylanase A	214.8 ^a	289.8 ^a	105.4 ^a	163.3 ^b	216.1 ^b	122.7 ^{ab}
Normal-ME wheat	Xylanase B	198.5 ^a	317.7 ^a	77.3 ^b	236.6 ^a	413.8 ^a	129.2 ^b
Normal-ME wheat	Xylanase C	157.0 ^b	219.5 ^b	89.0 ^{ab}	157.5 ^b	229.0 ^b	101.7 ^{ab}
Insoluble NSP							
Normal-ME wheat	Control	864.9 ^a	1299.0 ^a	547.1 ⁿ	764.8 ^a	1233.0 ⁿ	532.8 ^a
Normal-ME wheat	Xylanase A	727.7 ^b	1007.3 ^b	601.4 ⁿ	635.9 ^b	939.7 ^b	519.5 ^a
Normal-ME wheat	Xylanase B	689.9 ^b	984.0 ^b	449.6 ⁿ	800.4 ⁿ	1257.2 ^a	617.1 ^a
Normal-ME wheat	Xylanase C	848.4 ^a	1284.8 ^a	584.2 ^a	806.4 ^a	1315.6 ⁿ	552.8 ^a

Ara, arabinose; Xyl, xylose; Glc, glucose.

^{a,b,c,d} Values within a column with unlike superscript letters were significantly different ($P<0.05$).

* For details of diets and procedures, see Table 1 and p. 54.

Table 5. Main neutral sugar composition in jejunal and ileal digesta of broiler chickens fed diets containing low-metabolisable energy (ME) wheat with and without enzyme supplementation (g/kg marker)*
(Mean values)

	Enzyme	Jejunum			Ileum		
		Ara	Xyl	Glc	Ara	Xyl	Glc
Free sugars							
Low-ME wheat	Control	7.3 ^c	7.6 ^a	1365.6 ^a	6.6 ^a	5.2 ^a	781.2 ^a
Low-ME wheat	Xylanase A	31.7 ^a	80.5 ^a	1587.7 ^a	40.2 ^a	100.7 ^a	707.9 ^{ab}
Low-ME wheat	Xylanase B	9.8 ^a	15.0 ^a	1756.7 ^a	9.1 ^{bc}	12.9 ^{bc}	778.8 ^a
Low-ME wheat	Xylanase C	26.7 ^a	60.7 ^a	1547.4 ^a	18.3 ^a	40.0 ^a	582.6 ^b
Soluble NSP							
Low-ME wheat	Control	158.1 ^b	221.2 ^a	65.9 ^a	148.4 ^a	212.1 ^a	90.2 ^a
Low-ME wheat	Xylanase A	254.8 ^a	352.1 ^a	81.6 ^a	207.4 ^b	278.2 ^b	88.6 ^{ab}
Low-ME wheat	Xylanase B	247.5 ^a	372.6 ^a	75.8 ^a	262.8 ^a	431.4 ^a	98.9 ^a
Low-ME wheat	Xylanase C	160.5 ^b	199.1 ^b	66.5 ^a	150.5 ^a	199.4 ^c	71.3 ^b
Insoluble NSP							
Low-ME wheat	Control	903.6 ^a	1368.4 ^a	588.1 ^a	857.1 ^a	1405.3 ^a	580.2 ^a
Low-ME wheat	Xylanase A	790.9 ^a	1162.3 ^a	646.3 ^a	647.8 ^b	986.2 ^c	511.9 ^a
Low-ME wheat	Xylanase B	837.5 ^a	1255.2 ^a	607.3 ^a	729.7 ^b	1149.7 ^{bc}	539.3 ^a
Low-ME wheat	Xylanase C	815.3 ^a	1201.1 ^a	556.0 ^a	729.2 ^b	1188.8 ^a	497.2 ^a

Ara, arabinose; Xyl, xylose; Glc, glucose.

*^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

* For details of diets and procedures, see Table 1 and p. 54.

Discussion

The use of enzymes to increase growth rate and feed conversion efficiency in poultry by removing the anti-nutritive effect of NSP is well established. In addition to enhancing nutrient utilisation, the enzymes also offer other benefits. These include an overall decrease in the output of organic matter in the excreta due to the better utilisation of nutrients, a reduction in the moisture content of the excreta due to decreased gut viscosity, and an improvement in animal health due to the prevention of diseases associated with the proliferation of gut microflora. There is also an increased precision and flexibility in least-cost feed formulation due to the reduced variability in the nutritive value of ingredients. However, these claims are not applicable to all xylanases as it is thought that xylanases differ in their efficacy in degrading arabinoxylans, perhaps due to their substrate affinity.

The present study demonstrated a number of clear differences in the modes of action of different xylanases in wheat-based poultry diets. First, the differences in their ability to reduce the moisture content of the excreta were large, although wheat type had no effect on excreta moisture in the present study. For example, xylanase A reduced the moisture level of the excreta from 77% in the control diet to 73%. In the poultry industry, such a reduction means a change of the consistency of the excreta from a runny state to a dry, consistent form. Wet droppings are a big problem in the poultry industry, especially in laying hens where an increased percentage of dirty eggs is associated with wet and sticky excreta.

Second, to examine how xylanases with different modes of action exert their effect on the arabinoxylans in wheat, the present study measured the types and amounts of NSP present in various parts of the gut together with

Table 6. Duodenal, jejunal and ileal viscosity (mPa x s) of broiler chickens fed diets containing normal-metabolisable energy (ME) wheat and low-ME wheat with and without enzyme supplementation*
(Mean values and standard deviations)

Wheat	Enzyme	Duodenum		Jejunum		Ileum	
		Mean	SD	Mean	SD	Mean	SD
Normal-ME	Control	3.3 ^a	0.5	8.0 ^a	1.2	23.3 ^{bc}	4.0
Normal-ME	Xylanase A	2.2 ^d	0.3	4.1 ^d	0.7	6.8 ^a	1.1
Normal-ME	Xylanase B	3.6 ^{bc}	0.5	12.3 ^b	2.4	39.7 ^b	8.7
Normal-ME	Xylanase C	2.3 ^d	0.6	3.3 ^d	0.8	7.7 ^a	4.2
Low-ME	Control	4.0 ^b	0.8	9.4 ^a	0.8	28.3 ^b	5.8
Low-ME	Xylanase A	2.4 ^d	0.4	5.2 ^d	1.8	8.3 ^a	2.7
Low-ME	Xylanase B	4.7 ^a	0.8	18.4 ^a	4.5	84.1 ^a	42.6
Low-ME	Xylanase C	2.2 ^d	0.6	3.4 ^d	0.8	7.2 ^c	2.0
Probability of greater F value in ANOVA							
Wheat		***		***		**	
Enzyme		***		***		***	
Wheat x enzyme		NS		***		***	

*^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

** $P<0.01$, *** $P<0.001$.

* For details of diets and procedures, see Table 1 and p. 54.

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Table 7. Digestibility of insoluble and soluble NSP (iNSP and sNSP) in jejunal and ileal digesta of broiler chickens fed diets containing normal-metabolisable energy (ME) wheat and low-ME wheat with or without enzyme supplementation†
(Mean values)

Wheat	Enzyme	Jejunum		Ileum	
		iNSP	sNSP	iNSP	sNSP
Normal-ME	Control	0.07 ^b	-0.11 ^{ab}	0.14 ^{ad}	-0.09 ^{ab}
Normal-ME	Xylanase A	0.19 ^{ab}	-0.13 ^{ab}	0.28 ^{abc}	-0.17 ^{bc}
Normal-ME	Xylanase B	0.28 ^a	-0.21 ^{ab}	0.08 ^b	-0.73 ^d
Normal-ME	Xylanase C	0.06 ^b	-0.38 ^b	0.09 ^d	-0.15 ^{bc}
Low-ME	Control	0.26 ^a	-0.28 ^{ab}	0.25 ^{bc}	-0.07 ^{ab}
Low-ME	Xylanase A	0.29 ^a	-0.01 ^b	0.42 ^b	-0.28 ^a
Low-ME	Xylanase B	0.27 ^a	-0.24 ^{ab}	0.35 ^{ab}	-0.70 ^d
Low-ME	Xylanase C	0.30 ^a	-0.41 ^b	0.35 ^{ab}	0.02 ^a
Probability of greater F value in ANOVA					
Wheat		**	NS	***	NS
Enzyme		NS	**	*	***
Wheat x enzyme		NS	NS	NS	**

^{a,b,c,d,a,b} Values within a column with unlike superscript letters were significantly different ($P<0.05$).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

† For details of diets and procedures, see Table 1 and p. 54.

Table 8. Ileal and caecal volatile fatty acid (VFA) production (mmol/bird) in broiler chickens fed on diets containing normal-metabolisable energy (ME) wheat and low-ME wheat with or without enzyme supplementation†
(Mean values and standard deviations)

Wheat	Enzyme	Ileal VFA production		Caecal VFA production	
		Mean	SD	Mean	SD
Normal-ME	Control	12.6 ^{ba}	4.5	788.6 ^a	279.8
Normal-ME	Xylanase A	11.1 ^{bo}	3.6	699.5 ^a	131.8
Normal-ME	Xylanase B	11.5 ^{bo}	3.7	748.6 ^a	238.1
Normal-ME	Xylanase C	12.4 ^{bo}	4.8	513.4 ^a	187.4
Low-ME	Control	26.3 ^a	8.0	670.1 ^a	167.7
Low-ME	Xylanase A	12.9 ^{bo}	7.9	697.0 ^a	278.4
Low-ME	Xylanase B	19.5 ^{ab}	8.8	799.7 ^a	313.8
Low-ME	Xylanase C	10.1 ^c	4.5	561.0 ^a	270.1
Probability of greater F value in ANOVA					
Wheat		*	NS		
Enzyme		*	NS		
Wheat x enzyme		*	NS		

^{a,b,c} Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

* $P<0.05$.

† For details of diets and procedures, see Table 1 and p. 54.

changes in digesta viscosity in the relevant sections. This is because xylanases differing in their substrate affinity should have contrasting effects on digesta viscosity and their ability to release soluble NSP *in situ*.

There have been numerous studies exploring the mechanisms by which supplemental xylanases improve the nutritive value of wheat-based diets for poultry. Soluble arabinoxylans in wheat are generally believed to be responsible for the majority of the anti-nutritive activity of NSP in poultry by virtue of their capacity to increase intestinal viscosity and modulate gut microflora (Choct *et al.* 1996). It is well recognised that increased viscosity decreases the digestion and absorption rate in the lumen by changing gastrointestinal

functions (Edwards *et al.* 1988; Ikegami *et al.* 1990). In wheat-based diets, in particular, reduced performance is often related to an increase in intestinal viscosity (Bedford & Classen, 1992). Furthermore, increased intestinal viscosity alters lipid and protein digestibility (Wang *et al.* 1992). The addition of commercial xylanases capable of degrading soluble NSP reduces viscosity and improves the nutritive value of wheat fed to chickens (Choct *et al.* 1994; Veldman & Vahl, 1994; Dusel *et al.* 1998).

In the present study, xylanase A increased the soluble NSP levels in the jejunum of birds regardless of wheat type, and those in the ileum of birds fed the low-ME wheat. However, the increases in soluble NSP did not lead to elevated digesta viscosity, suggesting that whilst the enzyme attacked the insoluble cell walls of wheat to release soluble NSP into the digesta, it also effectively depolymerised the released NSP. On the other hand, xylanase B, a glycanase capable of attacking insoluble NSP, increased the soluble NSP levels in both the jejunum and ileum regardless of wheat type; the increases in soluble NSP levels, whilst comparable with that in birds fed diets containing xylanase A, were manifested in elevated digesta viscosity. This clearly suggests that xylanase B had no effect on the soluble NSP. As expected, xylanase C, an enzyme known to have affinity for only soluble NSP, effectively reduced digesta viscosity, presumably by cleaving the large molecules into smaller fragments. Since only high-molecular-weight soluble pentosans (molecular weight >500000) are responsible for increased digesta viscosity (Bedford & Classen, 1992), it was concluded that the released fragments had lower molecular weights. The finding that the concentration of free sugars in the ileum was elevated without a further increase in the concentration of soluble NSP indicates a continued depolymerisation of arabinoxylans throughout the intestine.

Third, the efficacies of the three xylanases in digesting the soluble and insoluble NSP were different. Xylanase A effectively degraded the insoluble NSP from the jejunum

to ileum while maintaining a similar 'digestibility' value for the soluble NSP fraction. This means that this enzyme was able to degrade the NSP to molecular sizes lower than approximately ten sugar units (otherwise the sugars would have ended up in the soluble NSP fraction). On the other hand, xylanase B solubilised the insoluble NSP fraction in both wheats, making the digestibility of the soluble fraction more negative. This indicates clearly that this enzyme changed only the insoluble NSP:soluble NSP fractions ratio by solubilising the insoluble NSP without degrading them to monomeric or oligomeric sugars. Interestingly, xylanase C reduced digesta viscosity, but appeared to release soluble NSP in the jejunum of birds fed both the normal- and low-ME wheats as indicated by the negative digestibility values. This finding suggests that enzyme C was only able to reduce the molecular size of the soluble NSP in the jejunum, but as the digesta moved down the digestive tract it continuously worked on the substrates, resulting in the digestibility coefficients of soluble NSP being higher in the ileum. Furthermore, even though xylanase C was assumed to have affinity for only the soluble NSP fraction, in the present study the digestibility of the insoluble fraction of the low-ME wheat, but not for the normal wheat, by this enzyme was comparable with the other two enzymes. This highlights the differences between the two wheats in their cell-wall structures, and hence their susceptibility to digestibility by the natural gut microflora or an enzyme that usually degrades soluble substrates. For example, the digestibility values for the insoluble NSP were 7 and 14 % in the jejunum and ileum, respectively, of birds fed the normal wheat, whereas the corresponding values were 26 and 25 % for birds fed the low-ME wheat. These data seem to suggest that a normal wheat and a low-ME wheat differ not only in their total contents of viscous polysaccharides, but also in their cell-wall matrices where they are more susceptible to solubilisation in the gut for the low-ME wheat, elevating the viscosity of the digesta.

As mentioned earlier, viscosity reduction by xylanases A and C and improvement in bird performance appeared to confirm the findings that viscosity is the major contributor to the anti-nutritive effect of wheat NSP in poultry (Choct & Annison, 1992b; Angkanaporn *et al.* 1994). However, the results related to the use of xylanase B, a viscosity-increasing enzyme, that also enhanced the performance of birds and tended to increase AME, contradict the conventional theory of viscosity. One explanation is that the improvement in performance and energy utilisation is most probably due to the breakdown of arabinoxylans-rich endosperm cell walls (Pettersson & Åman, 1989; Bedford, 2002) and the subsequent release of intracellular encapsulated nutrients. These data confirm the conclusion that the ability of NSP to increase digesta viscosity depends on their solubility, molecular size and tertiary structures (Fincher & Stone, 1982), and that quantitative measurements, such as the NSP level or digesta viscosity, should not be used as the sole indicator of the anti-nutritive activity of NSP in poultry diets.

The present results indicate that commercial glycanase products have beneficial effects in reducing the detrimental effects of wheat NSP both in low-ME and normal-ME

wheat. The effect of the enzyme products used in the present study on the degradation of NSP clearly highlights the differences between xylanases. To fully understand the modes of action of the different xylanase products in poultry diets based on wheat, more detailed analyses of structural changes of the NSP are necessary.

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